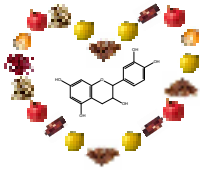

Optimization of the 4-Dimethylaminocinnamaldehyde Assay for Flavanols

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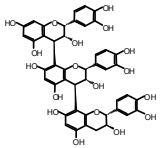
Abstract

Flavanols and their polymeric condensation products, proanthocyanidins represent the most common flavonoids consumed in the diet and are powerful antioxidants. Because of large degrees of chemical variation, isolation and quantification are difficult. The optimal conditions of the 4-dimethylaminocinnamaldehyde (DMAC) spectrophotometric assay for flavanols was evaluated to increase the sensitivity of the reaction and accuracy of the assay. The effects of acid nature (HCl and H2SO4), concentration (2, 4, 6, 8, and 10N), temperature (5, 15, 20, 25, 35, and 45ºC), reaction time (1, 2, 5, 10, 15, 20, and 25 min), sample water content (1.0, 2.0, 3.0, 4.0, 5.0, and 10%), DMAC concentration (1.0, 1.5, 2.0, 2.5, and 3.0%), and presence of interfering substances, were examined in order to develop a robust method for accurate assessment of flavanols. The use of sulfuric acid as acidulant in the reaction significantly improved the results. A mixture of 2% DMAC (w/v) in 1:1 methanol and 6N H2SO4 (v/v) showed maximum sensitivity when allowed to react for 12-15 minutes prior to analysis, with no further improvement shown by extending the time to the usual 20 minutes. The reaction of DMAC was most precise when conducted at constant temperature between 20 – 25ºC, with a sample water content ≤3%. Excess water caused loss of color in the reaction resulting on underestimation of the values. Optimization of the DMAC assay allows maximal sensitivity and detection of small concentration changes, giving a more accurate estimation of the healthy natural plant flavanols present in foods.

Background

Flavanols and their condensation products, proanthocyanidins (condensed tannins) are a group of plant polyphenols responsible for many astringent flavors in food products such as wine, chocolate, beer, pomegranate, and cranberry products. Flavanols / proanthocyanidins have been suggested to account for a significant fraction of the polyphenols ingested in the western diet (Santos-Buelga and Scalbert 2000). As the most popular flavonoids consumed in the diet (Aron and Kennedy 2008), flavan-3-ols are considered functional ingredients in many foods and beverages because of their positive effects on human health.

Recent studies have shown these compounds to contain strong antioxidant properties (Cos and others 2003), leading to a decrease in the instance of cardiovascular diseases (Porter and others 2001), and cancer (Santos-Buelga and Scalbert 2000). Most notably proanthocyanidins have received considerable attention because of their role in prevention of urinary tract infections (Foo and others 2000). Because of the wide variation in stereochemistry and polymerization amongst many flavan-3-ols, quantification methods have traditionally been cumbersome and inaccurate.



Traditional spectrophotometric assays such as the *n*-BuOH-HCl method utilize high acid and heat to hydrolyze proanthocyanidins into their monomeric forms prior to reaction with butanol. This procedure has been reported to show decreased reproducibility and increased variation. When hydrolyzing proanthocyanidins it is easy to underestimate total concentration because it is difficult to determine the degree of polymerization left after the cleavage reaction (Salunkhe and others 1990). Accordingly, the vanillin assay was developed to better accurately account for this predicament. The vanillin assay utilizes the aldehyde vanillin which reacts with meta oriented hydroxyl groups of the flavan-3-ol A-ring to produce a colored product at ~510 nm (Hageman and others 1997). However, in many fruit and vegetable products anthocyanins having a λ-max ~520 nm interfere with the assay.

A more modern method, the dimethylaminocinnamaldehyde (DMAC) assay, is analogous to the vanillin assay in its chemistry, but has improved accuracy and a λ-max - of ~640 nm which is outside of the spectra of anthocyanins, thus eliminating interference.

Several DMAC protocols have been developed for detection of flavan-3-ols, most notably those prepared by Li and others (1996) and Broeckling and Vivanco (2008).

The objective of this research was to develop the most robust, reliable, and accurate parameters for the DMAC assay. To accomplish this, a number of experimental parameters were studied including: acid nature and concentration of the DMAC reagent, reaction time, reaction temperature, sample water concentration, concentration of 4-dimethylaminocinnamaldehyde in the reagent, and interfering substances.

Methods

Preparation of Catechin Standards

Standard curves for the spectrophotmetric analysis were prepared by first dissolving 0.5 g catechin in methanol in a 100 mL volumetric flask. The volumetric flask was then sonicated for 30 seconds, inverted, and filled to its 100 mL capacity to ensure uniform distribution. Subsequent dilutions of 50, 100, 200, 300, 400, and 500 mg/L catechin in methanol were prepared by adding 1, 2, 4, 6, 8, and 10 mL of the catechin solution to methanol in order to achieve a final volume of 100 mL solution. Standard curves were prepared by adding 20 µL of each catechin standard to 2350 µL of methanol and 100 µL of the DMAC reagent in 3 mL disposable plastic cuvettes (path length = 1 cm). After reaction at room temperature (~20°C) for 12 min under minimal light conditions, each standard was subjected to spectrophotometric analysis using a UV-Vis Spectrophotometer (Shimadzu, Columbia, MD). Standards were replicated and analyzed 3 times at the reaction's visible absorption maximum (λmax) of 640 nm in order to ensure accuracy of the standard curve. Standards were plotted concentration (x-axis) vs. absorbance at 640 nm and subject to a regression analysis. All standard curves were required to have a r2 value of > 0.98 in order to be used in the analysis. Spectrophotometric readings were reported as catechin equivalents.

Preparation of DMAC Reagents

The DMAC reagent (4-dimethylaminocinnamaldehyde) was prepared immediately before use by dissolving 1.0, 1.5, 2.0, 2.5, or 3.0% DMAC powder (w/v) in a cold 1:1 mixture of methanol and 2, 4, 6, 8, or 10N HCl or H2SO4 (v/v). Reagents were kept in the freezer (-18°C) between analysis. New reagents were prepared daily because of the relative instability of the 4-dimethylaminocinnamaldehyde over long periods of time and sensitivity to light.

Reaction Studies

Using the prepared standards and reagents a spectrophotometric analysis of the DMAC-flavanol reaction was measured and analyzed at λ_{max} of 640 nm. All reactions unless specified below were subject to react with 2.0% DMAC in 6N H_2SO_4, for 12 min at room temperature (~20°C). The following variations in the assay were studied (n=3):

Acid Nature and Concentration – Use of 2, 4, 6, 8, and 10N HCl or H_2SO_4 in the preparation of the DMAC reagent. All standards were used in the construction of standard curves in this portion of the experiment.

DMAC Concentration – Use of 1.0, 1.5, 2.0, 2.5, and 3.0% DMAC on a (w/v) basis during the preparation of the reagent. Only 200 mg/L catechin standards were used in this study.

Reaction Temperature – The reaction was monitored at 5, 15, 20, 25, 35, and 45°C (± 0.5°C) to determine the influence of temperature. In this study, 200 mg/L catechin standards were covered with parafilm to prevent methanol evaporation at higher temperatures.

Reaction Time – The absorbance at λ_{max} of 200 mg/L catechin standards were recorded at 2, 5, 10, 12, 15, 17, 20, 25, 30, and 35 min

Water Content of the Sample – The absorbance at λ_{max} was measured when initial water contents of the 200 mg/L standard were prepared with 1.0, 2.0, 3.0, 4.0, 5.0, and 10.0% water in the sample. This was accomplished by substituting methanol during the standard preparation.

Interfering Substances – The interference of the following substances were tested by bringing 0.1 g each chemical to a 100 mL final volume in a volumetric flask, and then substituting the 20 µL of standard for 20 µL of the interfering substance solution.

Results

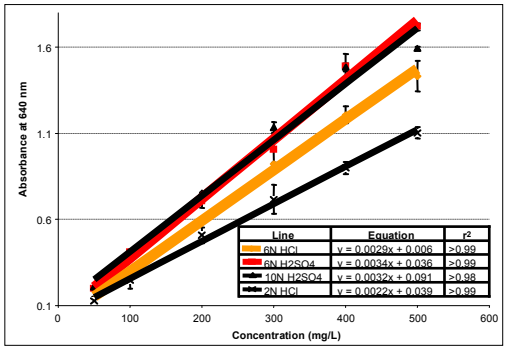


Figure 1. Concentrations ≥ 6N H_2SO_4 produce standard curves with an increased slope. 2N HCl, 6N HCl, 6N H_2SO_4, and 10N H_2SO_4 are shown as representative of both acids and their 5 individual concentrations. The 6N H_2SO_4 standard curve showed the greatest slope, indicating greater potential to accurately estimate the amount of catechin present. H_2SO_4 standard curves consistently showed higher slopes when compared to those of HCl. The slope of standard curves containing an acid concentration ≥ 6N did not significantly differ, but were consistently higher than the slopes of less concentrated standard curves. This is contrary and an improvement to the typical use of 6N HCl.

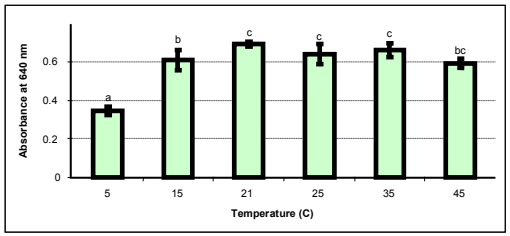


Figure 2. Temperature effects the reaction DMAC with 200 mg/L catechin. Consistent room temperature between 20 and 25°C should be used during the reaction. Both high and low temperatures had a significant effect on the absorbance at 640 nm after 12 min of reaction.

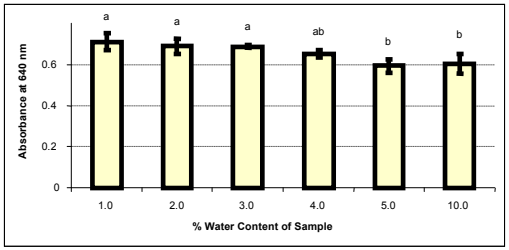


Figure 3. Water content of the sample greatly influences absorbance. A water content >3% significantly lowers the reaction absorbance at 640 nm, making the results of the assay less accurate. It is essential to minimize water content in food samples / extracts for accurate results. A consistent use of absolute methanol is recommended for the assay.

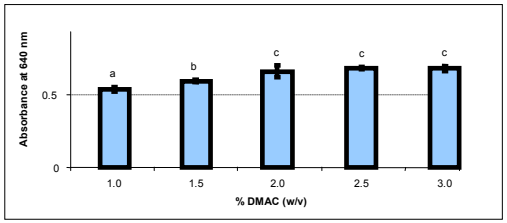


Figure 4. DMAC ≥ 2% (w/v) must be used to achieve maximum absorbance at 640 nm. Lower concentrations of DMAC did not fully react with the sample and thus showed lower absorbencies. Higher concentrations used showed no significant difference in the absorbance of the reaction. The displayed data shows the reaction of 200 mg/L catechin standard with the DMAC reagent.

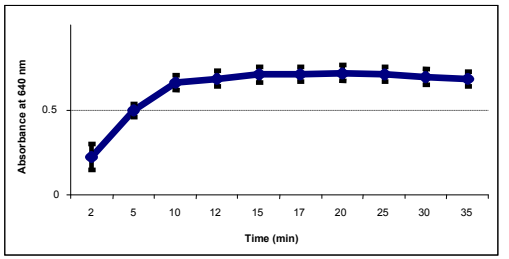


Figure 5. The DMAC – flavanol reaction shows optimum absorbance at 640 nm after 12 min. Measurements should be taken after 12-15 min. Prior to 12 min color formation and reaction of the DMAC reagent with 200 mg/L catechin standard was not optimal. Loss of color was also noted after 20 min, however this was not statistically significant. This is contrary to the typical reaction time of 20 min.

Conclusion and Discussion

- Critical evaluation of the 4-dimethylaminocinnamaldehyde (DMAC) assay for flavanols lead to a more accurate method for quantifying the healthy flavanol and proanthocyanidin compounds in food products.
- In order to achieve maximum sensitivity of the assay, optimum conditions for the DMAC assay included: 1) Use of 2% DMAC powder and 6N H_2SO_4 to prepare the DMAC reagent. 2) An optimum reaction time of 12-15 min at constant room temperature between 21 – 25ºC. 3) The use of absolute methanol in the assay and less than 3% water content of the sample.
- Interfering substances tested had no affect on the DMAC assay at 4 g/L concentration.

References

Sun B, Ricardo-da-Silva JM, and Spranger I. 1998. Critical factors of the vanillin assay for catechins and proanthocyanidins. J Agric Food Chem. 46: 4267-74.

Ferreira D, Slade D, Marais JPJ. 2006. Flavans and proanthocyanidins. In: OM Andersen and KR Markham. Flavonoids chemistry, biochemistry, and applications. Taylor & Francis. New York, NY.

Li YG, Tanner G, and Larkin P. 1996. The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. J Sci Food Agric. 70: 89-101.

Doronin CY, Chernova RK, and Gusakova NN. 2004. 4-dimethylaminocinnamaldehyde as a photometric reagent for primary aromatic amines. J Anal Chem 59(4): 377-87.

Acknowledgements

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